International Journal of Infectious Diseases 30 (2015) 52–56

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Absolute quantification of serum microRNA-122 and its correlation with liver inflammation grade and serum alanine aminotransferase in chronic hepatitis C patients

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ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form 1 September 2014
Accepted 22 September 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
MicroRNA-122
Chronic hepatitis C
ALT
Liver inflammation

SUMMARY

Objectives: MicroRNA-122 has been shown to be crucial for efficient HCV RNA replication in vitro. Pretreatment intrahepatic microRNA-122 levels in chronic hepatitis C (CHC) patients have been associated with the outcomes of interferon therapy. Here, we determined microRNA-122 serum levels in CHC patients and healthy donors using an absolute quantification approach and evaluated the correlation with liver inflammation grades and serum alanine aminotransferase (ALT) levels.

Methods: Serum samples were collected from 105 treatment-naive CHC patients, 11 acute hepatitis patients, and 33 healthy donors. Serum microRNA-122 was measured using the TaqMan RT-qPCR. The cycle threshold values were converted to copy numbers by drawing a standard curve using a chemical synthetic standard. For accurate quantification, copy numbers were further normalized according to the recovery ratios of spiked-in cel-miR-39.

Results: Serum levels of microRNA-122 were significantly higher in acute hepatitis and CHC patients than in healthy donors ($p < 0.001$). However, there was no significant association between microRNA-122 and ALT serum levels or liver inflammation grades.

Conclusions: The present study showed that serum microRNA-122 was elevated in acute and chronic hepatitis patients. However, this biomarker for acute liver injury did not reflect the liver inflammation activity in CHC patients.

1. Introduction

Hepatitis C is a global health problem. Approximately 130–200 million people are chronically infected with the hepatitis C virus (HCV).[1–3] Chronic hepatitis C (CHC) is a major cause of cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease. Different from hepatitis B, many CHC patients may remain asymptomatic or have mild symptoms for decades, even when overt end-stage liver disease has developed.[4,5] Alanine aminotransferase (ALT) serum levels remain the most commonly used marker for the severity of liver injury, but a chronic HCV infection may develop with or without ALT abnormalities.[6–8] Thus, it is of great importance to find new biomarkers for the early and accurate diagnosis of liver injury and disease progression in CHC patients.

Recently, studies have been performed to evaluate the potential of microRNAs as biomarkers for liver injury in various liver diseases.[9–26] Among these studies, microRNA-122 is of particular interest. MicroRNA-122 is an abundant liver-specific microRNA, accounting for approximately 70% of the total hepatic microRNA.[27] The HCV genome, in particular, contains microRNA-122 target sequences, and binding of microRNA-122 to the HCV genome positively regulates HCV replication.[28,29]

Since circulating microRNA-122 levels were first reported to predict drug-induced liver injury,[16] the potential of microRNA-122 as a biomarker for disease progression in viral hepatitis patients has been studied. For example, several clinical studies have shown that there is a significant correlation between microRNA-122 and serum ALT levels in chronic hepatitis B and C patients.[15,19,22,30]

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http://dx.doi.org/10.1016/j.ijid.2014.09.020

Moreover, microRNA-122 is also thought to be a serum marker candidate for viral hepatitis-related HCC.[17,26] However, a common problem in circulating microRNA studies is the diversity of the study population normalization methods, which gives rise to inconsistent or even conflicting results among these studies.[31]

In this study, we established a method for the absolute quantification of circulating microRNA-122. We then compared the serum levels of circulating microRNA-122 in CHC patients, acute hepatitis patients, and healthy donors, and evaluated the correlation between microRNA-122 and HCV RNA, serum ALT, and liver inflammation grade.

2. Methods

2.1. Patients

One hundred and five treatment-naive genotype 1 patients chronically infected with hepatitis C attending the 302 Military Hospital of China were enrolled in this study. Inclusion criteria were anti-HCV and HCV RNA-positive, and no previous interferon-based therapies. Those with HIV or hepatitis B virus (HBV) co-infections, or autoimmune diseases, were excluded. Serum samples were collected and stored at −80 °C until analysis.

Serum samples were also collected from 11 inpatients with acute hepatitis from the Peking University People's Hospital (six with acute hepatitis E, four with acute hepatitis B, and one with acute hepatitis A) and 33 healthy donors who were negative for hepatitis virus infection and had normal serum ALT levels.

This study was approved by the Ethics Committee for Human Experimentation at Peking University People's Hospital (Beijing, China) and was performed in accordance with the 1975 Declaration of Helsinki. Written informed consent was provided for sample collection and subsequent analysis.

2.2. Clinical chemistry, virology tests, and liver histology

ALT and aspartate aminotransferase (AST) were tested using an automated biochemical analyzer (model 7600; Hitachi, Tokyo, Japan) in the clinical laboratory of Peking University. Anti-HCV was tested using a chemiluminescent microparticle immunoassay (Architect; Abbott Laboratories, Chicago, IL, USA) and HCV was genotyped using restriction fragment length polymorphism (RFLP).[32] Hepatitis B virus surface antigen (HBsAg) and anti-HIV were detected using enzyme immunoassays (Abbott Laboratories, USA; and bioMérieux, Boxtel, the Netherlands, respectively). Serum HCV RNA levels were measured using the RealTime HCV Reagent (Abbott Laboratories, USA). Liver inflammation grades for the CHC patients were evaluated using the Ishak G scoring system.[33]

2.3. Serum total RNA extraction with spiked-in control microRNA mimic

To normalize the serum RNA extraction efficiency, a synthesized *Caenorhabditis elegans* microRNA mimic (Shanghai GeneChem, Shanghai, China), cel-miR-39, was used as the spike-in control. Cel-miR-39 (5 fmol) was added to each 200 μl of serum sample. Total RNA was isolated from serum using the mirVana PARIS Kit (Ambion, USA) following the manufacturer's protocol for the total RNA isolation procedure. Briefly, 200 μl of serum was mixed with an equal volume of 2× denaturing solution followed by organic extraction using acid–phenol and chloroform. The aqueous phase was mixed with 1.25 volumes of room temperature 100% ethanol. After washing three times, RNA was finally eluted using 100 μl 95 °C elution solution or nuclease-free water.

2.4. Reverse transcription and TaqMan real-time PCR assays of microRNA

Extracted RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems, USA). The reverse transcription reaction was as follows: 0.075 μl of 100 mM dNTP, 0.5 μl RT enzyme (50 U/μl), 0.75 μl 10× RT buffer, 0.094 μl RNAse inhibitor (20 U/μl), 2.5 μl eluted RNA, and 2.081 μl nuclease-free water. Reaction conditions were 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C until the end of the reaction.

Real-time PCR assays of the transcribed cDNA were performed using the TaqMan MicroRNA assays (Applied Biosystems, USA). The reaction system was as follows: 10.0 μl universal master mix II (2×), 1.0 μl 20× RealTime probes, 1.0 μl of cDNA, and 8.0 μl of nuclease-free water. Reaction conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

2.5. Generation of standard curves for absolute quantification of hsa-microRNA-122 and spiked-in control cel-miR-39

Synthetic hsa-microRNA-122 and cel-miR-39 (Shanghai GeneChem, Shanghai, China) were serially diluted to final concentrations of 200 nM, 20 nM, 2 nM, 0.2 nM, 0.02 nM, 2 pM, 0.2 pM, 0.02 pM, 2 fM, and 0.2 fM. hsa-microRNA-122 and cel-miR-39 serial dilutions were reverse-transcribed and assayed using real-time PCR analysis concurrently with RNA extracted from serum samples. Standard curves for hsa-microRNA-122 and cel-miR-39 were included on each plate of the miRNA TaqMan assays to convert the cycle threshold (Ct) values of each sample into the corresponding number of microRNA copies. Thus, the microRNA extraction efficiencies for each sample could be calculated by the yield ratios of added cel-miR-39, to obtain the absolute serum microRNA-122 quantification results.

2.6. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Comparisons of quantitative data were done using the Student's *t*-test or Spearman correlation analysis, as appropriate. All statistical tests were two-sided and a *p*-value of less than 0.05 was considered to indicate a statistically significant difference. The statistical software package used was GraphPad Prism v. 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Serum levels of microRNA-122 were significantly higher in acute hepatitis and chronic hepatitis C patients than in healthy donors

The characteristics of the study subjects are shown in Table 1. After converting the qPCR Ct values to copies per microliter of serum (copies/μl), microRNA-122 quantification results were further normalized by yielding ratios of added spike-in cel-miR-39. Finally, absolute quantification results of serum microRNA-122 were obtained. Median microRNA-122 serum levels were 4.017 $\log_{10}$ copies/μl (interquartile range (IQR) 3.756–4.327 $\log_{10}$ copies/μl) in healthy donors, 5.121 $\log_{10}$ copies/μl (IQR 4.874–5.537 $\log_{10}$ copies/μl) in CHC patients, and 6.577 $\log_{10}$ copies/μ1 (IQR 5.718–7.335 $\log_{10}$ copies/μl) in acute hepatitis patients. As shown in Figure 1, serum microRNA-122 levels were significantly higher in both acute hepatitis and CHC patients than in healthy donors ($p < 0.001$ and $p < 0.001$, respectively). Serum microRNA-122 levels were significantly higher in acute hepatitis patients than in CHC patients ($p < 0.001$).

Table 1
Patient characteristics

	Number, or mean ± SD
Chronic hepatitis C patients	
Age, years	
Mean ± SD	44.23 ± 9.18
Median	45
Range	20–64
Sex, male/female	70/35
ALT, U/L	
Mean ± SD	107.53 ± 245.06
Median	48
Range	5–1812
HCV RNA, IU/ml	
Mean ± SD	$6.12 \times 10^5 \pm 1.23 \times 10^6$
Acute hepatitis patients	
Age, years	
Mean ± SD	45.45 ± 14.24
Median	46
Range	23–68
Sex, male/female	8/3
Baseline ALT, U/L	
Mean ± SD	977.27 ± 394.12
Median	881
Range	399–1682
Healthy donors	
Age, years	
Mean ± SD	41.82 ± 7.29
Median	42
Range	30–54
Sex, male/female	20/13
ALT, U/L	
Mean ± SD	24.67 ± 8.60
Median	27
Range	11–38

SD, standard deviation; ALT, alanine aminotransferase; HCV, hepatitis C virus.

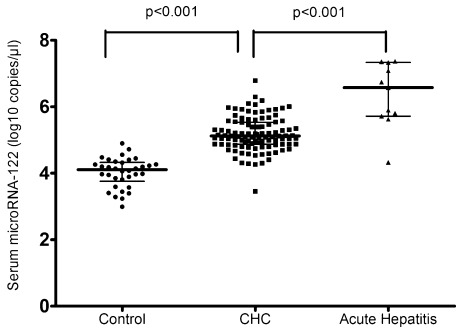

Figure 1. Serum levels of microRNA-122 were significantly higher in acute hepatitis and chronic hepatitis C patients than in healthy donors.

3.2. Serum microRNA-122 levels correlated with ALT levels in acute hepatitis patients

Serum ALT levels in acute hepatitis patients were assayed at baseline and weekly until the end of therapy. Among these patients the duration of therapy varied from 9 to 33 days. As shown in Figure 2A, there was a good correlation between serum microRNA-122 and baseline ALT levels in these acute hepatitis patients (Spearman $r = 0.8462$, $p < 0.001$). However, no significant correlation was observed for ALT and microRNA-122 levels at all time points combined, as shown in Figure 2B. Although serum ALT decreased rapidly during therapy, serum microRNA-122 levels stayed high, as shown in Figure 3. This suggests that dynamic changes in serum microRNA-122 levels in acute patients might be delayed in this population.

3.3. Serum microRNA-122 levels were not correlated with serum ALT levels, liver inflammation activity, or viral load in CHC patients

Of the CHC patients, 58.1% (61/105) had serum ALT below the upper limit of normal (ULN, 40 U/l). Liver inflammation activities in these CHC patients were assessed using the Ishak G scoring system. A strong correlation was observed between ALT levels and Ishak G scores (Spearman $r = 0.4386$. $p < 0.001$), as shown in Figure 4.

However, there was no significant correlation between serum microRNA-122 levels and ALT levels ($r = 0.07135$, $p = 0.4695$), or between serum microRNA-122 levels and Ishak G scores ($r = 0.08263$, $p = 0.4021$) (Figure 5A and B, respectively). Although microRNA-122 has been proposed as a biomarker candidate for liver injury in several studies, the absolute quantification results in our study showed that the serum level did not reflect the liver inflammation activity in CHC patients.

Similar to most previous studies, there was no significant correlation between serum microRNA-122 levels and HCV RNA levels ($r = 0.01692$, $p = 0.8639$); results are shown in Figure 5C.

4. Discussion

The liver-specific microRNA-122 has been shown to be crucial for efficient HCV RNA replication in vitro.[28,29] In addition, pretreatment intrahepatic microRNA-122 levels in CHC patients have been found to be related to the viral responses to interferon therapy.[34] Recently, circulating microRNA-122 has been considered a potential marker for liver injury and for clinical outcomes in

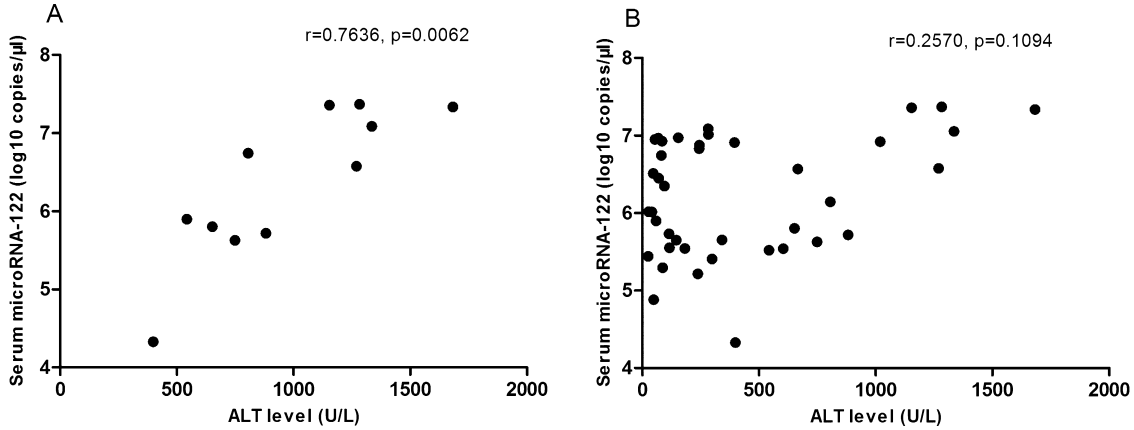

Figure 2. Correlation of serum microRNA-122 with alanine aminotransferase (ALT) levels in acute hepatitis patients. A, correlation between serum microRNA-122 and ALT levels at baseline in the acute hepatitis group; B, correlation between serum microRNA-122 and ALT levels at all time points in the acute hepatitis group.

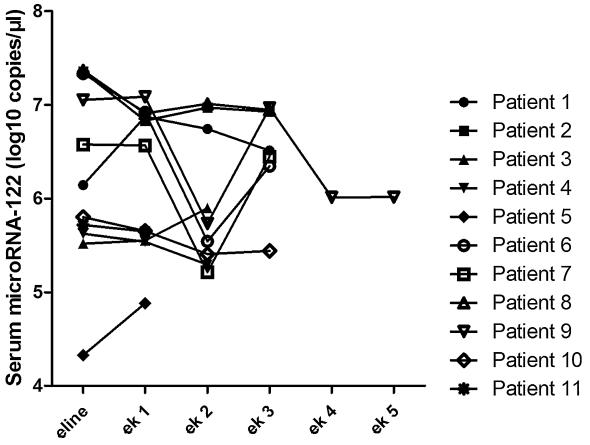

Figure 3. Dynamic changes in serum microRNA-122 in acute hepatitis patients. Serum microRNA-122 was at high levels during therapy (among the patients, the period of therapy varied from 9 to 33 days).

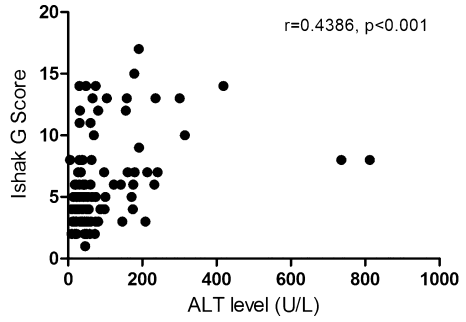

Figure 4. Correlation between alanine aminotransferase (ALT) levels and Ishak G scores in chronic hepatitis C patients. ALT levels were well correlated with Ishak G scores in chronic hepatitis C patients (Spearman $r = 0.4386$; $p < 0.001$).

hepatitis B, hepatitis C and related HCC. In the present study, we established an absolute quantification method for serum microRNA-122 assays. Results showed that serum microRNA-122 levels were elevated in both acute hepatitis and CHC patients. However, correlation between serum microRNA-122 and ALT levels was observed only in acute hepatitis patients and not in CHC patients. Serum microRNA-122, the candidate biomarker for acute liver injury, did not reflect the liver inflammation activity in CHC in our study.

The lack of correlation between serum microRNA-122 and ALT levels or liver injury that we found in our study is different from the results of many recent studies, with the exception of one study where no significant correlation was found between serum microRNA-122 and ALT levels in hepatitis B patients.[35] As mentioned above, there were problems involved in the circulating microRNA studies, which caused inconsistent or even conflicting results in similar studies. Circulating microRNA-122 is no exception. Bihrer et al.[15] found that serum microRNA-122 levels were comparable between CHC patients with persistently normal ALT levels and healthy volunteers. However, another study[19] observed an 11.6-fold higher microRNA-122 level in CHC patients with normal ALT levels compared with healthy controls. Qi et al.[17] found that microRNA-122 serum levels were significantly higher in HCC than in chronic hepatitis B patients, while the opposite result was also reported.[26] It was reported recently that serum microRNA-122 levels markedly decreased while ALT significantly increased with increasing fibrosis stage during CHC infection.[36] There are also different opinions on the predictive value of baseline serum microRNA-122 levels for the responses to antiviral therapy in CHC patients.[20,37]

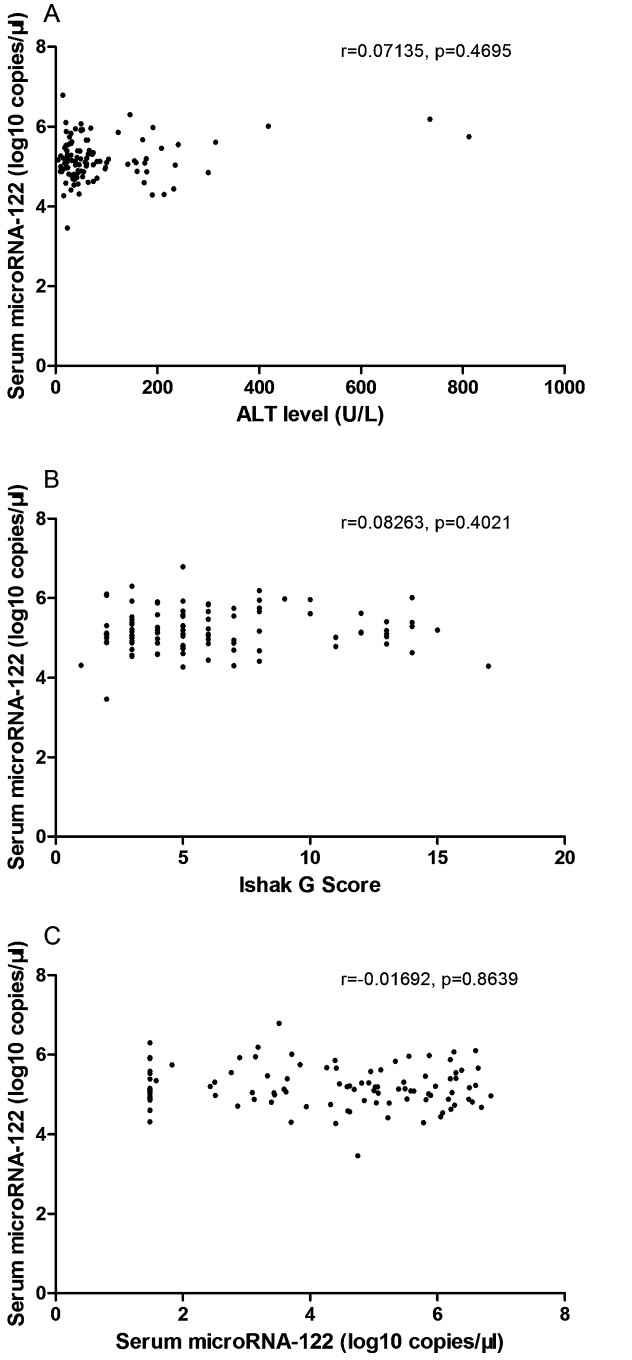

Figure 5. Correlations of serum microRNA-122 levels with (A) alanine aminotransferase (ALT) levels, (B) Ishak G scores, and (C) HCV RNA levels in chronic hepatitis C patients.

These derivations might be due to the different sample sizes or races of study populations. However, a general problem with the circulating microRNA assays is the lack of normalization of the results. There are currently no commonly accepted internal references for circulating microRNAs. Different methods are used to normalize the circulating microRNA quantification results. U6, for example, which has frequently been used as the internal control for serum microRNAs, has been shown to be unstable in the serum of chronic hepatitis B and HCC patients.[38] Thus, an inappropriate internal control may affect the accuracy of quantification. In some studies, no internal controls were used, and circulating microRNA levels were simply indicated as the Ct values. Thus, the accuracy of

circulating microRNA quantitation results may be affected by inadequate normalization of data. Most studies have used a relative quantification method instead of an absolute one, which will, to some extent, limit the final clinical application of promising microRNA candidates.

In the present study, we performed absolute quantification of microRNA-122 levels to determine the microRNA-122 copy numbers in the serum. The use of cel-miR-39 as a spike-in control helped to normalize the serum RNA extraction efficiency, which improves the reliability of the results. Results showed that there was no correlation between serum microRNA-122 levels and ALT or Ishak G scores in CHC patients. Considering the limited sample size, validation of these results requires a larger number of clinical samples. The absolute quantification results showed the correlation between microRNA-122 and baseline ALT levels in acute hepatitis patients. However, serum microRNA-122 remained at high levels while ALT decreased to normal levels. In future studies, we will investigate the correlation between microRNA-122 and ALT in acute liver injury rat models to determine whether the slow decrease in serum microRNA-122 level is due to the elimination half-life of microRNAs or if there are more complicated regulatory mechanisms involved.

In conclusion, absolute quantification of serum microRNA-122 showed that serum microRNA-122 levels were significantly higher in acute hepatitis and CHC patients than in healthy donors. However, there was no significant correlation between the serum microRNA-122 levels and ALT levels or liver inflammation grades in CHC patients.

Acknowledgements

This work was supported by grants from the National Science and Technology Major Project for Infectious Diseases Control during the 12th Five-Year Plan (grant number 2012ZX10002003), National S&T Basic Work Program of China (grant number 2013FY113900), and Project of Beijing City Science and Technology Nova (2011013).

Ethical approval: This study was approved by the Ethics Committee for Human Experimentation at Peking University People's Hospital (Beijing, China), and was performed in accordance with the 1975 Declaration of Helsinki. Written informed consent was provided for sample collection and subsequent analysis.

Conflict of interest: There are no conflicts of interest regarding this study.

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